DETECTION BY POLYMERASE CHAIN REACTION OF ENTEROVIRUS-LIKE SEQUENCES IN PATIENTS WITH EPIDEMIC NEUROPATHY IN CUBA

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SUMMARY
Enterovirus-like sequences were found in patients with epidemic neuropathy (EN) in Cuba. We examined cerebrospinal fluids (CSFs) and nerve biopsy or necropsy specimens for the presence of Enterovirus RNA by reverse transcriptase-polymerase chain reaction (RT-PCR). Specific amplification was detected in 40 out of 111 CSFs from patients with EN and in 1 out of 13 control CSFs. In the two optical nerves examined from patients with EN, positive signals were obtained by RT-PCR. A cytopathic effect (CPE) was shown in the CSF from patients positive by RT-PCR and in 43 negative ones. Two of these agents caused enterovirus-like (Cox A9) CPE and the rest showed a CPE atypical of enteroviruses. The possible role of enteroviruses in the etiology and pathogenesis of EN is not known.

RESUMEN
Se detectaron secuencias homólogas con enterovirus en pacientes con neuropatía epidémica (EN) en Cuba. Se examinaron muestras de líquido cefalorraquídeo (CSFs), biopsias de nervios y especímenes de necropsias para la presencia de ARN de enterovirus empleando la reacción de reverso-transcripción-reacción en cadena de la polimerasa (RT-PCR). Se detectó amplificación específica en 40 de las 111 muestras de CSFs de pacientes con EN y en una de 13 controles. En dos nervios ópticos de pacientes con EN examinados se detectó ARN homólogo a enterovirus mediante RT-PCR. Se demostró la presencia de efecto citopático (CPE) en los CSFs positivos por RT-PCR y en 43 muestras que resultaron negativas por RT-PCR. Dos de estos agentes causaron un CPE típico de enterovirus (CoxA9) y el resto mostró un CPE diferente al característico para enterovirus. El papel de enterovirus en la etiopatogenia de la EN no se conoce.

INTRODUCTION
During the second half of 1991, 1992 and the beginning of 1993 an epidemic of a neuropathic disease characterized by affection of the nervous system producing central and peripheral symptoms appeared in different regions of Cuba (1). At the beginning of the epidemic the patients complained of weight loss, blurred vision, hypersensitivity to light and gradual loss of visual acuity over a period of approximately 1 to 4 weeks. The ophthalmological examination showed central or cecocentral scotoma, pallor of the temporal edge of the optic disk and loss of axonal fibers in the papillo-macular tract; this clinical picture was characterized as optic neuropathy. Other cases complained of pain in the upper and lower limbs, paresthesia mainly in the legs, burning sensation on the soles of the feet and other subjective symptoms. On clinical examination they showed a loss in bilaterally and symmetrical sensitivity to vibration on touch, diminished or absent Achilles reflexes, very intense patellar reflexes and absence of Babinski’s sign. Those findings were consistent with sensory neuropathy, including dorsolateral myelopathy. During the epidemic, three forms of the clinical picture were identified: purely ocular (optic neuropathy), purely peripheral neuropathy and mixed cases.

The disease was termed epidemic neuropathy (EN) with about 50,000 cases diagnosed up to October 1993. Many different hypotheses about the etiology of the disease were suggested; nutritional deficit and a possible toxic agent were the most commonly accepted but not yet proved. Nevertheless, the possibility of an infectious agent (namely a virus) acting in association with toxic and nutritional factors was considered and evaluated from the beginning of the epidemic.

During multicenter and multidisciplinary studies performed by the Cuban Ministry of Health, the Laboratory of Virology of the Institute "Pedro Kouri" (Havana, Cuba),
succeeded in the isolation of viruses from the cerebrospinal fluid (CSF) in several patients affected with different clinical pictures of EN. Similar results were independently obtained in another series of patients in the Center for Genetic Engineering and Biotechnology and the AIDS Reference Center in Havana. In the first ten cases, two types of cytopathic effect (CPE) were observed: in two cases, an enterovirus-like CPE was observed, and in the rest of the samples, a weak, slowly progressing and delayed CPE was observed in cultured cells. The typical enterovirus-like CPE was characterized as a Coxsackie virus A9 by neutralization tests with the LBM pool of sera and by partial nucleotide sequence of two isolates (2).

In order to investigate the presence of enterovirus sequences in the central nervous system (CNS) of patients with EN we examined CSF and tissue samples (biopsy and necropsy) from peripheral and central nerves of patients with this syndrome by the RT-PCR. We carried out the investigation in two independent laboratories, the laboratory of Virology from the Institute "Pedro Kour" (IPK) and the laboratory of Molecular Virology from the Center for Genetic Engineering and Biotechnology (CIGB).

**MATERIALS AND METHODS**

**Patients and specimen collection**

CSFs were obtained from 111 patients with EN. Those CSFs were aliquoted in a laboratory in which enteroviruses (EVs) had not been manipulated previously. We obtained sural nerves by biopsy from six patients, and tissues of optical nerves were recovered from two patients with EN who died of causes not related to the disease (accident and heart stroke). As controls, CSFs obtained from different patients without any symptoms or signs associated with the EN as confirmed previously by clinical and neurological examination were included (surgical patients (10), aseptic meningitis (1) and AIDS associated cryptococcosis (2)). Nervous tissues were obtained by sural biopsy (indicated for the etiological diagnosis of their specific diseases) in one case of flaccid paralysis and two cases of myopathy. Two optical nerves from AIDS deceased patients were also obtained, formalin-fixed and paraffin embedded. All specimens were kept at -70°C before use.

**RNA isolation, cDNA obtention and polymerase chain reaction**

The RT-PCR procedure was performed as previously described with some slight modifications (3). Briefly, 100 µL aliquots of CSF of clinical and control specimens were treated with RNAse (Promega Corp., Madison, Wisconsin, USA) and viral RNA was extracted by the addition of sodium dodecyl sulfate to a final concentration of 2.5%, followed by the addition of 1 volume of phenol:chloroform (1:1 mixture). Extracted RNA was precipitated with 0.3 M sodium acetate pH 5.3 and 2.5 volumes of cold 100% ethanol during 20 minutes at -70°C and 30 min at -20°C and resuspended in 10 µL of double-distilled water.

The RNA extraction from the biopsy of sural nerves was done as follows: The biopsy (about 20 mg) was ground and resuspended in 100 µL of a lysis buffer containing 20 mM Tris-HCl pH 8.0, 20 mM EDTA, 2% sodium dodecyl sulfate and 500 µg/mL Proteinase K and incubated for 4-6 hours at 56°C. Nucleic acids were purified by two phenol extractions followed by two precipitations with isopropanol (5 min each at 4°C) in the presence of 0.3 M sodium acetate pH 5.3. Samples were centrifuged at 12 000 rpm for 5 min at 4°C, the supernatant removed and the pellet air-dried.

We performed the nucleic acid extraction from the necropsy with the same protocol. However, an initial step for the elimination of paraffin with xylene-ethanol was carried out (4).

Oligomeric primers both downstream and upstream and an internal probe were chosen from the highly conserved 5' end of the non-coding region of the enterovirus genome as described (3). These oligonucleotides were synthesized as single-stranded DNA, using an automated synthesizer (Gene Assembler Plus, Pharmacia-LKB, Sweden).

The reverse transcriptase reaction was performed with 5µL of each RNA sample with the addition of 40 U of RNasin, 4 µL of 5X reverse transcriptase buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 50mM MgCl2, 50 mM DTT, 2.5 mM spermidine), 4 µL of 1.25 mM dNTP mixture, 1mL of the down-stream primer (20 pmol), 10 µL of Avian Reverse Transcriptase (Promega) and double-distilled water to complete 20 µL. The mixture was then incubated for 30 min at 42°C.

One quarter of the total cDNA reaction was used for the PCR and the following reagents were added: 5 µL of 10X PCR buffer (500 mM KCl, 100 mM Tris-HCL pH 9 and 1.0% Triton), 3 µL 15 mM MgCl2, 8 µL of 1.25 mM dNTP mixture, 1 µL of each oligonucleotide (50 pmol), 0.2 µL of T. aquaticus DNA polymerase (5 U/µL, Promega) and double-distilled water to complete 50 µL. The reaction was covered with 100 µL of mineral oil. The amplification cycles were performed in a thermocycler apparatus (Gene Ataq, Pharmacia, Sweden).

The first cycle was at 95°C for 3 minutes and was followed for 40 cycles of 1 min denaturation (94°C), 1 min annealing at 55°C and 1 min primer extension at 72°C. The last step of primer extension was extended to 12 min. The amplification product was detected by gel electrophoresis and Southern blot analysis using the internal oligonucleotide end labelled with 32P. Samples were considered positive if a positive hybridization signal at estimated size of 154 bp was detected.

Each experiment was repeated at least twice to confirm a positive result. We evaluated the sensitivity of the procedure in our conditions using a previously titrated poliovirus stock and tested the specificity using RNA extracted from VERO and Hela cells and from different enteroviruses, rubella virus, hepatitis A virus, varicella zoster virus, herpes simplex virus type 1, hepatitis B virus and cytomegalovirus as substrate for RT-PCR.

The precautions for avoiding PCR contamination included physical separation of the process for obtaining RNA, cDNA and PCR, pre- aliquoted reagents, UV irradiation of the PCR master mix and a piece of cotton at the top of the tips for avoiding the contamination of the pipettes. Those precautions were rigorously applied and controlled in both laboratories in which the research was conducted. Controls including those previously mentioned for primer specificity and the performance of RT-PCR with distilled water, tissue culture media, fetal calf serum and PBS were included to check for contaminations.
Additionally, different sets of primers for the amplification of herpes simplex (5), varicella (6) and cytomegalovirus (7) were synthetized and used to analyze the presence of those viruses in the CSFs of patients with EN.

RESULTS

The performance of the RT-PCR was evaluated in the laboratory with viral RNAs representing the four enteroviral groups. About 70 plaque forming units (PFU) of poliovirus 1 were detected after Southern blot hybridization (data not shown). Except for echovirus 22 (lane 15 in figure 1), tested enteroviruses were detected by the appearance of the expected 154 bp fragment on electrophoretic analysis (figure 1). With the primers employed in these experiments, we did not expect to detect echovirus 22 and it was included as an additional control. Negative controls were performed with RNA extracted from VERO (figure 2) and HeLa cells (data not shown) and with distilled water, tissue culture media, fetal calf serum, PBS, rubella virus, hepatitis A virus, varicella zoster virus, herpes simplex virus type 1, hepatitis B virus and cytomegalovirus (figure 1). These samples were clearly negative, showing the absence of contamination and the specificity of the primers for enteroviral sequences (figure 1). PCR products were blotted onto nylon membranes and probed with the radiolabeled internal oligomer. In each case in which we observed the 154 bp fragment, the oligomer hybridized to the same band and negative controls remained negative after the Southern blot analysis (figure 2).

Specific PCR amplification was detected after Southern blot hybridization in 40 out of 111 CSFs from patients with EN (table 1). All these CSFs showed cytopathic effect when inoculated into Vero cells (Rodriguez and Mas, unpublished results). Two of these CPEs corresponded to enterovirus-like CPE and were identified as Coxsackievirus A9 by neutralization tests with the LBM pool of sera and by partial nucleotide sequence (8). The rest of the CSFs found to be positive by RT-PCR (n=38) or negative (n=33) showed the weak CPE in Vero

Fig.1 RT-PCR analysis of six enterovirus serotypes and negative controls. Reaction products were separated in a 1.5 % agarose gel. The following enteroviruses (approximately 10^3 TCID_{50} of each) were analyzed: coxsackievirus A9 (lane 5), coxsackievirus B4 (lane 6), poliovirus 1 (lane 7), echovirus 4 (lane 8), echovirus 22 (lane 15) and coxsackievirus B6 (lane 16). Negative controls were analyzed and applied in the following order: distilled water (lane 1), tissue culture media (lane 2), fetal calf serum (lane 3), PBS (lane 4), herpes simplex type 1 virus (lane 9), rubella virus (lane 10), hepatitis A virus (lane 11), varicella zoster virus (lane 12), cytomegalovirus (lane 13) and hepatitis B virus (lane 14).
cells non-typical of enteroviruses. Thirty-eight samples that were found negative by RT-PCR were not analyzed in cell culture. One out of 13 control CSFs showed the presence of enteroviral RNA by RT-PCR. All the sural nerve biopsies from six patients with EN were negative by RT-PCR. Samples from optical nerves belonging to two patients with EN who died during the disease for other causes were consistently positive (figure 2). Nervous tissues of different origins obtained as negative controls from cases without the disease, were found negative.

The different attempts that were made with the sets of primers for herpes simplex, varicella and cytomegalovirus trying to amplify those viruses from patients CSF were negative.

**DISCUSSION**

Neuropathy syndromes associated with severe malnutrition, alcoholism or as a consequence of infectious diseases have been described and published elsewhere (9). Between 1955 and 1970, about 10 000 cases of subacute myelo-optic neuropathy (SMON) were diagnosed in Japan. Clinical characteristics of the EN in Cuba largely resembled the characteristics of SMON, except for the absence of abdominal pain referred by the Japanese patients that commonly preceded neurological manifestations (10). A drug (cloquinol) was considered as a possible cause of the epidemic (11). A virus (virus of Inoue-Melnick) was isolated at the end of the epidemic (12) but the role of this agent remains obscure. The Cuban epidemic of neuropathy had some new characteristics, the most important being the extension and the number of cases that were reported in a short period of time (1). The possibility of a virus involved in the etiology of EN in Cuba was

![Fig.2 RT-PCR analysis of CSF samples and optical nerves from patients with EN. Reaction products were separated in 1.5% agarose gels (upper panel) and analyzed in Southern blots (lower panel) employing as hybridization probe a 32P-end-labeled oligonucleotide homologous to the conserved 5'non-coding region of the enterovirus genome (ref.3). Lanes: (1), HaeIII-digested pBR322 plasmid DNA (DNA size markers); (2), reaction with RNA from poliovirus type 1 (positive control); (3), reaction with RNA from Vero cells (negative control); (4), CSF from a control individual; (5), CSF from a patient with EN; (6-7), reactions with RNA from the optical nerve of patients with EN.](image)

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*The number of cases that were positive or negative by RT-PCR for the presence of the enterovirus-like sequences were included.

*Laboratory of Virology, Institute "Pedro Kouri". bCenter for Genetic Engineering and Biotechnology. cCerebral Spinal Fluids. dDescription of control group can be found in Material and Methods. eThe positive case correspondence
suggested in early 1992 during the beginning of the epidemic outbreak, and enteroviruses were soon isolated from CSFs of patients with EN (2). To investigate this fact further we examined CSFs and nervous tissues from patients with the disease by means of an RT-PCR using primers for the enterovirus 5’ non-coding region. Using this methodology we were able to detect enterovirus sequences in 36.03% of the CSFs from clinical cases and in 7.69% of the CSFs from the control group. In the study of tissues, only the two optical nerves from patients with EN were positive and the rest of the samples from the patients and control groups were negative. The positivity of the RT-PCR in the two optical nerves of patients with EN had the drawback that the enterovirus-like sequences could be found in the surrounding tissues and not in the optical nerve. Other techniques like in situ PCR need to be applied in order to confirm this result.

The RT-PCR had been recommended for the detection of poliovirus and other enterovirus infections in CSF and muscle biopsy specimens because of its sensitivity (4,13). The equivalent of about 10 viral genomes can be detected using serial dilutions of a cDNA clone (13). Under our assay conditions, the RT-PCR detected 70 PFU when a titrated stock of poliovirus was serially diluted in normal CSF (data not shown).

The prevalence of enteroviral infections of the CNS producing meningitis or encephalitis have been documented (14), but the pathogenic potential of enteroviruses for the CNS is not fully understood (14,15,16). Enteroviruses have been suggested as possible etiological agents in amyotrophic lateral sclerosis (ALS) due to the similarity in pathological changes and tissue tropism in diseases caused by those viruses (15). It was an intriguing finding that one of the 13 controls showed enteroviral sequences on his CSF. This patient was clinically examined after we obtained this result and has remained asymptomatic until now. However, as a possible multicausal disease, virus infection does not necessarily need to correlate with clinical symptoms. Previous studies have suggested the ability of enteroviruses to persist at low levels within the CNS (17) and more recently it has been described that the polyomavirus JCV and BKV frequently establish a latent CNS infection (18).

Detection of enterovirus RNA in 36% of CSF from patients having EN by RT-PCR was an unexpected finding and raised new questions about the significance of viral infections of the CNS and the role of these viruses in the EN in Cuba.

The role of enteroviruses in the etiology and pathogenesis of EN remains unknown.

However, since only two isolates resulted to be coxsackieviruses, it is tempting to speculate that viruses of the group of coxsackie are not related to the EN. How an enterovirus (or an entero-like virus) can act in the etiology of the disease as a unique factor or most likely associated to other factors like nutritional imbalance, toxic agents, stress or an immuno compromised status calls for an answer in the future. The possibility that other infectious agents (namely a virus) that could be present in the CSF of the patients producing the non-typical CPE needs to be investigated, and work is currently in progress to address these questions.

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